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(54) Title  
FUNCTIONALLY SPECIFIC ANTIBODIES

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(57) Claim

1. A composition comprising two or more different antibody species, each having a diagnostic or therapeutic agent attached thereto, wherein each of said antibody species is reactive with a different epitope on a target site and wherein the patterns of cross-reactivity for each antibody species are non-overlapping, for use within a method for delivering one or more diagnostic or therapeutic agents to a target site within a mammalian or human host.

3. The composition of claim 2 wherein the diagnostic agent is a diagnostically effective radionuclide.

8. The composition of any of claims 1, 2 or 7 wherein each therapeutic agent is selected from the group consisting of therapeutically effective radionuclides, drugs, toxins, sensitizers, and biological response modifiers.

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COMMONWEALTH OF AUSTRALIA  
PATENTS ACT 1952  
COMPLETE SPECIFICATION

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COMPLETE SPECIFICATION FOR THE INVENTION ENTITLED:

Functionally specific antibodies

The following statement is a full description of this invention, including the best method of performing it known to me/us:-

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1 dose that may be administered is lowered. Delivery of  
diagnostic agents to normal cross-reactive tissues may  
3 result in misdiagnosis.

A need remains for antibodies of improved specificity  
5 for target tissue such as tumors and decreased cross-  
reactivity with non-target (e.g., normal) tissues. This  
7 would be accomplished by identification of absolutely  
tumor-specific antigens and antibodies or by improved  
9 immunization techniques to yield totally tumor-specific  
antibodies. It is possible, however, that such target  
11 tumor-specific antigens do not exist, and that antibodies  
having the desired degree of specificity therefore will  
13 never be isolated.

#### 15 Summary of the Invention

The present invention provides a method of delivering  
17 one or more diagnostic or therapeutic agents to a target  
site within a mammalian or human host, comprising adminis-  
19 tering to said host two or more different antibody species,  
each having one of said agents attached thereto, wherein  
21 each of said antibody species is reactive with a different  
epitope on the target site and wherein the patterns of  
23 cross-reactivity for each antibody species are non-  
overlapping. Each of the antibody species may have a  
25 diagnostic agent attached thereto. Alternatively, the  
antibody species may have the same or different thera-  
27 peutic agents (e.g., radioisotopes, toxins, or drugs)  
attached thereto. In one embodiment of the invention,  
29 each antibody species is a monoclonal antibody reactive  
with a cancer cell.

31 Also provided by the present invention is a method of  
producing additive accumulation of two or more immunocon-  
33 jugates on a target tissue within a human or mammalian  
host while minimizing additive accumulation of the  
35 immunoconjugates on non-target tissues, comprising:

- a) administering a first immunoconjugate comprising a  
37 first antibody species to the host;

1 (b) administering one or more additional immunocon-  
jugates each comprising a different antibody  
3 species to the host, wherein each of the antibody  
species reacts with a different epitope on the  
5 target tissue and the different antibody species  
have non-overlapping patterns of cross-reactivity.

7 The present invention also provides a method of admin-  
istering two or more different therapeutic agents to a  
9 human or mammalian host to eradicate target cells, wherein  
each therapeutic agent is administered at or near its  
11 maximum tolerated dose to the host, while minimizing  
toxicity toward non-target tissue comprising attaching  
13 each different therapeutic agent to a different antibody  
species, wherein each antibody species reacts with a  
15 different epitope on the target cells, and wherein the  
patterns of cross-reactivity for each antibody species are  
17 non-overlapping, and administering each of the resulting  
immunoconjugates at or near the maximum tolerated dosage  
19 to the host.

Use of antibody species having non-overlapping cross-  
21 reactivity in accordance with the present invention  
provides advantages which include reduced chances of  
23 misdiagnosis (in the case of diagnostic agents) and  
reduced toxicity toward non-target tissues (in the case of  
25 the therapeutic agents).

#### 27 Brief Description of the Figures

29 Figure 1 depicts the binding of three monoclonal antibodies  
with small cell lung cancer cells, as determined by flow  
31 cytometry.

#### 33 Detailed Description of the Invention

The present invention provides methods for delivering  
35 diagnostic or therapeutic agents to a desired target site  
within a human or mammalian host. The agents are attached  
37 to two or more different antibody species which are

1 reactive with different epitopes (on the same or different  
2 antigens) on the target site but which have non-overlapping  
3 patterns of cross-reactivity. An epitope is an antigenic  
4 determinant, and a given antigen may comprise more than  
5 one epitope. Thus, the different antibodies (along with  
6 the agents attached thereto) accumulate additively on the  
7 desired target site, while only one antibody species  
8 accumulates on each type of cross-reactive non-target  
9 tissue. Additive accumulation of two or more of the  
10 immunoconjugates on non-target tissues thus is minimized  
11 or eliminated. A higher percentage of the administered  
12 agent therefore becomes localized in vivo on the target  
13 site compared to the non-target tissues. In the case of  
14 diagnostic agents, target sites can be more clearly  
15 detected or imaged against a comparatively lower  
16 "background" of the agent on non-target sites, and the  
17 incidence of misdiagnosis may be reduced as a result. For  
18 therapeutic agents, the comparatively lower amount of  
19 agent delivered to non-target sites results in reduced  
20 toxicity toward normal tissues.

21 As discussed above, antibodies with 100% specificity  
22 to a desired target site have yet to be isolated, in spite  
23 of the significant effort that has been directed toward  
24 that goal. The only possible exceptions are anti-idiotypic  
25 antibodies, but any such antibody is specific for the  
26 B-lymphoma cells of only one individual, and thus must be  
27 separately developed and isolated for each new patient.  
28 The use of two or more antibodies specific for a target  
29 site but with non-overlapping cross-reactivity to normal  
30 tissues, in accordance with the present invention, pro-  
31 vides a method of increasing the proportionate amount of  
32 antibody-bound agent(s) that become localized at a target  
33 site compared to non-target sites, even though a single  
34 antibody having such increased target specificity has not  
35 been isolated.

36 As used herein, the statement that the patterns of  
37 cross-reactivity for each of the antibody species are

1 non-overlapping means that the list of non-target tissues  
2 to which one antibody species binds is substantially dif-  
3 ferent from the list of non-target tissues to which the  
4 second antibody species binds. If a third antibody species  
5 is to be administered to the same patient, an antibody is  
6 used which has a pattern of cross-reactivity that is sub-  
7 stantially different from that of both the first and the  
8 second antibody species. The patterns of cross-reactivity  
9 are to be different enough to produce the desired results  
10 of the method of the invention, namely, proportionately  
11 less of the agent on non-target tissues such that back-  
12 ground is reduced (in the case of diagnostic agents) and  
13 toxicity to normal tissues is reduced (in the case of  
14 therapeutic agents). The desired results may still be  
15 achieved in some cases when the patterns of cross-  
16 reactivity for the different antibody species include a  
17 very small number of the same non-target tissues. For  
18 example, two antibodies may both cross-react with a  
19 non-target cell type (e.g., normal T-cells) which is not  
20 essential to the health of the patient, so the desired  
21 reduction in toxicity to the patient is achieved in spite  
22 of additive accumulation of therapeutic agents bound to  
23 these antibodies on the non-essential cell type. However,  
24 it is preferable to choose antibody species which do not  
25 cross-react with any of the same non-target tissues.

26 The method of the present invention generally begins  
27 with identification of the two or more antibodies to be  
28 employed. As discussed above, antibody species which bind  
29 to the desired target site but which have negligible or no  
30 overlapping cross-reactivity to non-target sites are chosen  
31 for use.

32 The antibody species employed in the present invention  
33 may be intact antibody molecules, fragments thereof, or  
34 functional equivalents thereof, including genetically  
35 engineered variations thereof. Examples of antibody  
fragments are  $F(ab')_2$ ,  $Fab'$ ,  $Fab$ , and  $Fv$ , produced by

1 conventional procedures. While polyclonal antibodies may  
2 be employed in the present invention, monoclonal antibodies  
3 (MAbs) are preferred. In one embodiment of the invention,  
4 the MAbs are directed against a tumor-associated antigen  
5 in humans. Many monoclonal antibodies directed against  
6 specific target sites (e.g., cancer cells) in vivo have  
7 been developed. Examples of such MAbs are anti-TAC, or  
8 other interleukin-2 receptor antibodies; 9.2.27 and  
9 NR-ML-05 to a 250 kilodalton human melanoma associated  
10 proteoglycan; NR-LU-10 to 37-40 kilodalton pancarcinoma  
11 glycoprotein; and OVB<sub>3</sub> to an as yet unidentified  
12 cancer-associated antigen.

13 Known methods such as those of Kohler and Milstein  
14 (Eur. J. Immunol., 6:292 (1976)) may be used to generate  
15 additional monoclonal antibodies reactive with a desired  
16 antigen. Monoclonal antibodies to tumor-associated  
17 antigens have been produced by several methods. One  
18 method is described in U.S. Patent No. 4,172,124 and  
19 another different method is described in co-pending U.S.  
20 patent application serial number 773,340, entitled "A  
21 Method for Improving the Elicitation of IgG Class  
22 Monoclonal Antibodies to Tumor-Associated Antigens and  
23 Glycoproteins".

24 The patterns of cross-reactivity for MAbs directed  
25 against a particular target site are analyzed to identify  
26 a set of two or more target-specific MAbs with non-  
27 overlapping cross-reactivity which may be used for a given  
28 diagnostic or therapeutic purpose. The antibodies produced  
29 may be screened by several methods. Advantageously, the  
30 in vitro testing procedure used to determine reactivity  
31 with tumors and cross-reactivity with normal tissues is  
32 immunohistochemical analysis. By immunohistochemical  
33 methods, the tissues (both normal and tumor tissues) to  
34 which the antibody in question binds are identified by  
35 exposing the tissue to the antibody and then detecting the  
36 presence of the antibody after washing to remove unbound  
37 antibody. Cryostat sections (i.e. frozen tissue sections

1 produced as described in Example I below) are preferred  
2 since fixation may destroy particular antigens and is  
3 associated with uncertain differences in timing of  
4 fixation that may result in varying degrees of antigen  
5 preservation. Nonetheless, if a particular antigen is  
6 known to be preserved by fixation, then fixed tissues may  
7 also be used in the in vitro testing procedure.

8 Procedures for conducting in vitro immunohistochemical  
9 analyses are known. See, for example, Ceriani et al.,  
10 Cancer Research, 47:532-540, January 15, 1987. Another  
11 suitable in vitro assay is presented in Example I below.  
12 Thus, the normal tissue cross-reactivity of antibodies  
13 that are reactive with the desired target site may be  
14 evaluated in such assays, and two or more suitable anti-  
15 bodies are chosen for use as functionally specific  
16 antibodies.

17 The term "functionally specific antibodies" as used  
18 herein refers to two or more different antibodies which  
19 react with different epitopes on a particular target site,  
20 and have non-overlapping patterns of cross-reactivity with  
21 normal tissues. The use of two or more functionally  
22 specific antibodies in diagnostic or therapeutic procedures  
23 results in increased specificity toward target tissues  
24 compared to diagnostic or therapeutic agents comprising  
25 only one antibody or antibodies having similar patterns of  
26 cross-reactivity toward normal tissues, as described above.

27 Functionally specific antibodies react with the same  
28 tumor(s), but they need not react with all of the same  
29 cells in those tumors. For example, if two antibodies  
30 reacted with a separate population of tumor cells within  
31 the same tumor they could additively deliver radiation to  
32 that tumor. Functionally specific antibodies, however,  
33 will more commonly bind to overlapping populations of  
34 tumor cells.

35 Functionally specific antibodies must, however, not  
36 bind to all the same normal tissues. The less overlap  
37 there is in binding to normal tissues, the more



1 functionally specific the antibody pairs. Any lack of  
2 overlap improves the tumor specificity compared to a  
3 single antibody alone, but a completely functionally  
4 specific antibody pair preferably will have no overlap or  
5 will overlap only on a nonessential organ or cell type.  
6 Examples of functionally specific antibodies include the  
7 monoclonal antibodies designated NR-LU-10 (also referred  
8 to as TFS-2) and NR-LU-11 (also referred to as TFS-4) that  
9 both react with small cell lung cancer, but both only  
10 cross react with thyroid although each alone reacts with  
11 several other normal tissues, as described in Example I  
12 below. Another example is NR-ML-05 and anti-G<sub>D3</sub> anti-  
13 bodies that both react with melanoma, but both cross-react  
14 with no known normal tissue in common although each alone  
15 exhibits cross-reactivity with several normal tissues.

16 In one embodiment of the invention, the same diagnostic  
17 agent is attached to each of the different antibody  
18 species. Any suitable known diagnostic agent may be  
19 employed, including but not limited to radioisotopes such  
20 as <sup>99m</sup>Tc, <sup>111</sup>In, <sup>123</sup>I, <sup>131</sup>I, <sup>76</sup>Br, or <sup>18</sup>F, nuclear  
21 magnetic resonance imaging contrast agents, and the like.  
22 The radionuclides generally will be in the form of a  
23 stable complex, e.g., a chelate. The biodistribution of  
24 such diagnostic agents in vivo may be analyzed by appro-  
25 priate standard external (i.e., non-invasive) means. A  
26 preferred diagnostic agent is the radionuclide metal  
27 <sup>99m</sup>Tc. Following administration of a <sup>99m</sup>Tc-labeled  
28 antibody, the biodistribution of the radionuclide metal  
29 may be detected by scanning the patient with a gamma  
30 camera using known procedures. Accumulations of <sup>99m</sup>Tc  
31 diagnostic agent at target sites are thus easily imaged.

32 In another embodiment of the invention, each antibody  
33 species has the same or a different therapeutic agent  
34 attached thereto. Any suitable known therapeutic agent  
35 may be used, including but not limited to therapeutically  
36 effective radionuclides, drugs, toxins, and biological  
37 response modifiers. The choice of agent will depend on

1 the type of disease to be treated (i.e., the type of  
target cells). Such radioisotopes include, among others,  
3  $^{188}\text{Re}$ ,  $^{186}\text{Re}$ ,  $^{203}\text{Pb}$ ,  $^{212}\text{Pb}$ ,  $^{212}\text{Bi}$ ,  $^{109}\text{Pd}$ ,  $^{64}\text{Cu}$ ,  $^{67}\text{Cu}$ ,  
 $^{131}\text{I}$ ,  $^{211}\text{At}$ ,  $^{97}\text{Ru}$ ,  $^{105}\text{Rh}$ ,  $^{198}\text{Au}$ , and  $^{199}\text{Ag}$ . The radio-  
5 nuclides generally are in the form of stable complexes  
such as chelates, which may be prepared by known methods.

7 Examples of toxins which may be employed are ricin,  
abrin, diphtheria toxin, Pseudomonas exotoxin A, ribosomal  
9 inactivating proteins, and mycotoxins; e.g., trichothecenes.  
Trichothecenes are a species of mycotoxins produced  
11 by soil fungi of the class fungi imperfecti or isolated  
from Baccharus megapotamica (Bamburg, J.R., Proc. Molec.  
13 Subcell Bio. 8:41-110, 1983; Jarvis & Mazzola, Acc. Chem.  
Res. 15:338-395, 1982). Therapeutically effective modified  
15 toxins or fragments thereof, such as those produced through  
genetic engineering or protein engineering techniques, may  
17 be used.

Any suitable therapeutic drug may be employed,  
19 depending on the nature of the patient's illness. Among  
the many therapeutic drugs that have been used to treat  
21 various forms of cancer are nitrogen mustards such as  
L-phenylalanine nitrogen mustard and cyclophosphamide,  
23 intercalating agents such as cis diamino dichloro platinum,  
antimetabolites such as 5-fluorouracil, vinca alkaloids  
25 such as vincristine, and antibiotics such as adriamycin  
and bleomycin.

27 Drugs known to enhance the cytotoxic effect of certain  
anti-cancer drugs and radiotherapeutic agents also may be  
29 used. Such drugs are commonly referred to as sensitizers.  
The sensitizing drug may be attached to one antibody  
31 species and a radionuclide or appropriate anti-cancer drug  
attached to another antibody species, for example.

33 Among the sensitizers known to enhance the therapeutic  
effectiveness of radiation are metronidazole, misonidazole,  
35 certain 2-sulfamyl-6-nitrobenzoic acid derivatives,  
2,6-disubstituted derivatives of 3-nitropyrazine, and  
37 certain isoindoleione compounds. (See U.S. Patents Nos.

1 4,647,588; 4,654,369; 4,609,659; and 4,494,547.) Examples  
of sensitizers which enhance the activity of various  
3 therapeutic drugs (e.g., anti-cancer drugs) are buthionine  
sulfoximine, calcium channel blockers such as verapamil,  
5 and diltiazem. (See U.S. Patent No. 4,628,047 and  
Important Advances in Oncology 1986, DeVita et al., Eds.,  
7 J.B. Lippincott Co., Philadelphia, pages 146-157 (1986).  
One skilled in the art to which this invention relates  
9 will be able to identify appropriate combinations of  
sensitizers and therapeutic agents.

11 Examples of biological response modifiers are inter-  
ferons (alpha, beta, and gamma), tumor necrosis factor,  
13 lymphotoxin, and interleukins (IL-1, -2, -3, -4, -5, and  
-6).

15 The procedure for attaching an agent to an antibody  
will vary according to the chemical structure of the agent.  
17 Antibodies are proteins which contain a variety of func-  
tional groups; e.g., carboxylic acid (COOH) or free amine  
19 (-NH<sub>2</sub>) groups, which are available for reaction with a  
suitable functional group on an agent molecule to bind the  
21 agent thereto. Alternatively, the antibody and/or agent  
may be derivatized to expose or attach additional reactive  
23 functional groups. The derivatization may involve attach-  
ment of any of a number of linker molecules such as those  
25 available from Pierce Chemical Company, Rockford, Illinois.  
(See the Pierce 1986-87 General Catalog, pages 313-354.)  
27 A bifunctional linker having one functional group reactive  
with a group on a particular agent, and another group  
29 reactive with an antibody, may be used to form the desired  
immunoconjugate. Alternatively, derivatization may  
31 involve chemical treatment of the antibody; e.g., glycol  
cleavage of the sugar moiety of the glycoprotein antibody  
33 with periodate to generate free aldehyde groups. The free  
aldehyde groups on the antibody may be reacted with free  
35 amine or hydrazine groups on an agent to bind the agent  
thereto. (See U.S. Patent No. 4,671,958.) Procedures for  
37 generation of free sulfhydryl groups on antibodies or

1 antibody fragments also are known. (See U.S. Patent No.  
2 4,659,839.) Many procedures and linker molecules for  
3 attachment of various compounds including radionuclide  
4 metal chelates, toxins and drugs to proteins such as  
5 antibodies are known. See, for example, European Patent  
6 Application Publication No. 188,256; U.S. Patents No.  
7 4,671,958; 4,659,839, 4,414,148; 4,699,784; 4,680,338;  
8 4,569,789; and 4,590,071; and Borlinghaus et al. (Cancer  
9 Research, 47:4071-4075, August 1, 1987).

10 A problem associated with some methods of linking  
11 certain therapeutic compounds to antibodies is that the  
12 biological activity of the compound (e.g., drug, toxin,  
13 etc.) may be reduced when the compound is attached to the  
14 antibody. When a therapeutic agent is conjugated to the  
15 antibody through a stable covalent bond, for example,  
16 release of the agent in its free, maximally active form at  
17 the target site generally would not be expected to occur.  
18 Therefore, immunoconjugates comprising linkages which are  
19 cleavable in the vicinity of the target site may be used  
20 when the desired activity of the agent would be diminished  
21 if not released from the antibody. Cleaving of the linkage  
22 to release the agent from the antibody may be prompted by  
23 enzymatic activity or conditions to which the immunocon-  
24 jugate is subjected either inside the target cell or in  
25 the vicinity of the target site. When the target site is  
26 a tumor, a linker which is cleavable under conditions  
27 present at the tumor site (e.g., when exposed to tumor-  
28 associated enzymes or acidic pH) may be used.

29 A number of different cleavable linkers have been  
30 described previously. See U.S. Patents Nos. 4,618,492;  
31 4,542,225; and 4,625,014. The mechanisms for release of  
32 an agent from these linker groups include by irradiation  
33 of a photolabile bond, and acid-catalyzed hydrolysis.  
34 U.S. Patent Application Serial No. \_\_\_\_\_ (attorney  
35 docket number 6922.476) filed December 2, 1987, entitled  
36 "Cleavable Immunoconjugates for the Delivery and Release  
37 of Agents in Native Form," discloses immunoconjugates

1 comprising linkers of specified chemical structure, wherein  
the linkage is cleaved in vivo, releasing the compound  
3 (radiotherapeutic agent, drug, toxin, etc.) in its native  
form. The linker is susceptible to cleavage at mildly  
5 acidic pH, and is believed to be cleaved during transport  
into the cytoplasm of a target cell, thereby releasing the  
7 biologically active compound inside a target cell. U.S.  
Patent No. 4,671,958 includes a description of  
9 immunoconjugates comprising linkers which are cleaved at  
the target site in vivo by the proteolytic enzymes of the  
11 patient's complement system. In view of the large number  
of methods that have been reported for attaching a variety  
13 of radiodiagnostic compounds, radiotherapeutic compounds,  
drugs, toxins, and other agents to antibodies, one skilled  
15 in the art will be able to determine a suitable method for  
attaching a given agent to an antibody.

17 The antibody-agent conjugates prepared as described  
above are administered to a human or mammalian host in  
19 diagnostically or therapeutically effective amounts. The  
amounts will vary depending on such factors as the anti-  
21 bodies used, since antibodies vary with respect to the  
number of receptors on the target cells and their affinity  
23 for the receptors. The dosage also will vary according to  
the agent used, as toxins and drugs, for example, vary  
25 with respect to their potency. It will be evident to one  
skilled in the art how to determine the optimal effective  
27 dose for a particular immunoconjugate. Procedures for  
determining the maximum tolerated dose for therapeutic  
29 agents, e.g., cytotoxic agents, also are known. Of  
course, since two or more different antibody species are  
31 used to deliver agents in vivo, the total dosage adminis-  
tered is the sum of the agents on all the different anti-  
33 body species administered to the patient.

For many treatment methods currently in use, toxicity  
35 caused by the action of a therapeutic agent on normal  
tissues has been a dosage-limiting factor. Thus, dosages  
37 which would be more effective in eradication of target

1 cells (e.g., cancer cells) could not be safely given due  
2 to the side effects caused by this toxicity. One of the  
3 advantages of the method of the present invention is that  
4 additive accumulation of therapeutic agent(s) on target  
5 cells occurs without additive accumulation of the agent(s)  
6 on cross-reactive normal tissues, due to the non-  
7 overlapping cross-reactivities. Thus, the total dosage  
8 administered may be increased to improve therapeutic effec-  
9 tiveness without increasing the undesirable side effects.  
10 The present invention thus provides an improved method for  
11 treatment of illness such as cancer when compared with  
12 other methods that employ a single antibody or a mixture  
13 of antibodies having overlapping cross-reactivities.

14 In diagnostic procedures, improved results may be  
15 achieved without increasing the dosage above conventional  
16 dosages. For example, target sites may be more accurately  
17 and effectively imaged due to the greater contrast between  
18 target and non-target tissues because of the non-additive  
19 binding of a diagnostic agent to non-target sites. Second,  
20 some metastases may express one antigen more than another.  
21 This provides the ability to target different metastases  
22 preferentially. Sequential administration of diagnostic  
23 imaging agents allows confirmation of sites of accumulation  
24 as true positives.

25 In therapeutic procedures, two or more antibody  
26 species, each having a different therapeutic agent attached  
27 thereto, may each be administered to a patient at the  
28 maximum tolerated dose, since each normal tissue type will  
29 bind only one of the immunoconjugates and therefore will  
30 not be exposed to the additive effects of both agents.

31 In another embodiment of the present invention, the  
32 antibodies may be covalently joined. One method is to  
33 link the Fab' fragment of one antibody species to the Fab  
34 or Fab' of the other. The hybrid F(ab')<sub>2</sub> would have  
35 bivalent binding to the target site but only univalent  
36 binding to any cross-reactive antigen. After adminis-  
37 tration of the hybrid antibody conjugated to a diagnostic

1 or therapeutic substance, unconjugated native bivalent  
antibody could be used to displace the hybrid antibody  
3 from normal tissues where it has only univalent binding  
potential, and therefore lower affinity.

5 The present invention also provides a kit for diag-  
nostic or therapeutic use comprising two or more antibody  
7 species wherein each of the antibody species is reactive  
with a different epitope on a target site and the patterns  
9 of cross-reactivity for each of said antibody species are  
non-overlapping. Thus, a particular kit contains func-  
11 tionally specific antibodies reactive with a desired  
target site such as a particular cancer site. The  
13 antibody species in a kit will vary according to the  
desired target site; e.g., whether the target cells are  
15 melanoma cells, SCLC cells, etc. Depending on the intended  
use of the antibodies, diagnostic or therapeutic agents  
17 may be attached to the antibodies, as described above.  
The antibodies in the kits may already have the agents  
19 attached thereto. Alternatively, the user (e.g., medical  
personnel) may attach the desired agent(s) to the anti-  
21 bodies before use.

In one embodiment of the invention, each antibody  
23 species in the kit has a chelating compound attached  
thereto. The chelating compound is capable of chelation  
25 of a diagnostically or therapeutically effective radio-  
nuclide metal. One kit of the present invention comprises  
27 two monoclonal antibody species designated NR-LU-10 and  
NR-LU-11, or fragments thereof, which bind to cancer cells.

29 The following examples are provided for purposes  
of illustration, not limitation.

31

**EXAMPLE I. Two Antibodies Against Small Cell Lung Cancer**

33

Three antibodies (KS 1/4, TFS-2 and TFS-4) that react  
35 with small cell lung cancer were recently described. See  
Varki, N.M., Reisfeld, R.A., and Walker, L.E., "Antigens  
37 Associated with a Human Lung Adenocarcinoma Defined By

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1 Monoclonal Antibodies." Cancer Res 44:681-87 (1984);  
Okabe T., Kaizu T., Fujisawa M. et al. "Monoclonal  
3 Antibodies to Surface Antigens of Small Cell Carcinoma of  
the Lung" Cancer Res 44:5273-78 (1984). Extensive  
5 evaluation on normal tissues by immunohistochemistry  
reveals that TFS-2 and KS 1/4 exhibit cross-reactive  
7 binding to normal thyroid, pancreas, hepatic ducts, and  
epithelial tissues, while TFS-4 binds to normal nerve  
9 tissues, adrenal glands, a subpopulation of circulating  
lymphocytes and thyroid gland. Both bind strongly and  
11 additively to small cell lung cancer cell lines. The in  
vitro testing procedure is as follows.

13 In summary, the assay comprises reacting murine mono-  
clonal antibodies with antigens expressed on different  
15 cell type surfaces. Rabbit-anti-mouse antibodies conju-  
gated to horseradish peroxidase then react with the murine  
17 antibodies. The peroxidase enzyme reduces hydrogen  
peroxide to water in the presence of 3,3'-diaminobenzidine .  
19 (DAB), and a positive reaction product is indicated by a  
brown stain on the tissue. Monoclonal antibodies which  
21 are not of murine origin may be used in the test procedure  
provided that an appropriate secondary antibody conjugated  
23 to horseradish peroxidase is available for use.

One fact to be noted is that some tissues exhibit  
25 endogenous peroxidase staining. One tissue slide should  
be stained with DAB only to serve as a control for  
27 endogenous staining. A hematoxylin/eosin (H/E) stained  
section aids in the identification of different cell  
29 types. Appropriate negative control proteins are run with  
each set of serial sections tested with antibodies.

31 The slides are kept level during the entire staining  
procedure. Reagents should cover the entire tissue  
33 section and should not pool at either end of the slide.  
Slides which are not level and reagents which pool in one  
35 area of the tissue during staining will give inaccurate  
results. Specific time periods are assigned to each step  
37 of the staining procedure. Attention should be directed



1 to timing of each staining step. Care should be taken not  
to scrape tissue during staining. Frozen sections may be  
3 fragile and should be handled gently and with care.

The reagent 3,3'-diaminobenzidine (DAB) maybe prepared  
5 in advance. DAB is available in 5.0 gram quantities. The  
DAB preparation should be done under a laminar flow hood  
7 while wearing single use medical gloves. To 5.0 grams DAB  
20.0 ml HPLC grade water is added, and the DAB is  
9 dissolved. 200 ul of the DAB solution is transferred into  
each glass bottle until all of the solution has been used,  
11 and the bottles are placed in freezer boxes. The uncapped  
bottles are covered with several layers of utility wipers.  
13 The DAB solution is lyophilized in bottles for 2 days. The  
bottles then are removed from the lyophilizer, capped and  
15 frozen at -70°C until ready for use.

Reconstitution of lyophilized DAB is accomplished by  
17 removing 1 vial of DAB from -70°C freezer and allowing it  
to warm to room temperature on bench top. The assay for  
19 each monoclonal antibody then is conducted as follows:

In hood: add 5.0 ml phosphate buffered saline pH 7.0  
21 without calcium/magnesium (PBS) to vial via needle and  
syringe. Pump solution up and down through syringe until  
23 DAB has dissolved. Filter entire DAB solution through a  
0.45 micron filter into 95 ml PBS. DAB should be made  
25 fresh with every immunoperoxidase test and should not be  
reconstituted more than 30 minutes prior to use. 0.03%  
27 H<sub>2</sub>O<sub>2</sub> activates the DAB.

Filter approximately 15 ml of chicken serum through a  
29 115 ml 0.45 micron filter. The chicken serum must be  
filtered daily for use in frozen section staining. Make a  
31 5% solution of chicken serum in PBS (PBS-CS). Make  
appropriate dilutions of test antibodies (5 ug/ml is an  
33 appropriate dilution for most test and control  
antibodies). Also prepare rabbit and anti-mouse (RaM)  
35 conjugate 1/50 in PBS-CS plus 4% human serum type AB.

Spin solutions in an ultracentrifuge for 1 hour at 100,000  
37 x g at 4°C. Note that RaM conjugate must be spun daily.

1 Conjugate not used in making the 1/50 working dilution  
should be discarded. Do not save. It is not always  
3 necessary for primary test antibodies to be spun (e.g.,  
supernatants may be used as is).

5 The test antibodies are contacted with samples of  
various normal human tissues which have been fixed onto  
7 glass slides previously. The procedure for preparation of  
the fresh frozen tissue specimens bound to slides is as  
9 follows, using a variable temperature cryochamber and  
microtome (available from Cryostat):

- 11 1. Frozen tissue/OCT mold is affixed to cutting chuck  
with liquid OCT which quickly freezes at -20°C.
- 13 2. Mount chuck to cryo-microtome chuck holder.
3. Orient chuck for proper sectioning.
- 15 4. Tissue is sectioned at 4-6 microns.
5. Sections are mounted to dry glass microslides  
17 (which have been previously subbed with an aqueous  
5% gelatin solution and allowed to dry.
- 19 6. Glass microslides with tissue sections are then  
fixed in cold acetone (precooled to -20°C) for 10  
21 minutes.
7. Fixed tissue slides are then placed in a 37°C  
23 incubator to thoroughly evaporate the acetone.

If not used immediately, the tissue-bearing slides may  
25 be prepared for storage as follows:

1. Place (dry) acetone fixed slides in plastic  
27 microslide holder; replace lid.
2. Wrap box with aluminum foil.
- 29 3. Put foil wrapped box in plastic zip-lock bag with  
2-3 desiccant packets - seal shut with minimal air  
31 space.
4. Store in -70°C freezer.
- 33 5. Prior to use, allow acetone fixed slides to reach  
room temperature. Slides should be completely  
35 dry. Rehydrate slides in PBS for 10 minutes.
6. The test antibodies are contacted with the  
37 tissue-bearing slides as follows:

1 Incubate slides for 20 minutes with 100 ul PBS with 5%  
2 chicken serum (PBS-CS) containing 4% pooled normal rabbit  
3 serum to block non-specific binding. Rinse slides with  
4 PBS using 500ml squirt bottle. Incubate sections with 100  
5 ul test antibody (undilute or at appropriate dilution) to  
6 assess antigen expression or with 100ul PBS-CS only to  
7 visualize endogenous murine immunoglobulin. Rinse slides  
8 with PBS. Agitate in two fresh PBS washes in large beakers  
9 for 5 and 10 minutes respectively. Incubate each slide  
10 with 100 ul rabbit-anti-mouse immunoglobulin conjugated to  
11 horseradish peroxidase (RAM-HRPO) diluted 1/50 in PBS-CS  
12 plus 4% human serum for 30 minutes. Rinse sections with  
13 PBS and agitate in three fresh 5-minute PBS washes.

14 Incubate slides collectively in DAB 0.5 mg/ml with  
15 0.03% hydrogen peroxide for 10 minutes. Rinse in PBS  
16 bath. Counterstain slides for 1 to 1-1/2 minutes with  
17 Mayer hematoxylin. Rinse in PBS bath. Dip slides in a  
18 saturated lithium carbonate aqueous solution used for  
19 bluing. Rinse in PBS bath. Dehydrate sections with 5  
20 minute incubations in 30%, 60%, 75% 200° ethanol, 200°  
21 ethanol, and xylene respectively. Mount sections with a  
22 small amount of Permount and a #1 micro coverglass. Slides  
23 may be stored for observation under the microscope or saved  
24 for further reference.

25 As previously described, a positive reaction is indi-  
26 cated by a brown reaction product. A negative reaction  
27 slide will appear to be blue. Slides are scored for  
28 percent of cells stained, intensity of the stain, and  
29 homogeneity of the stain. The types of tissue with which  
30 a particular antibody reacts are thereby identified.

31 The above-described patterns of cross reactivity for  
32 the three antibodies were thereby determined. A different  
33 procedure was used to test reactivity of these antibodies  
34 toward small cell lung cancer (SCLC) cell lines designated  
35 SHITE-1. TSP-4 and KS-14 react with different antigens.  
Each reacts with small cell lung cancer.

1     The antibodies were incubated at 4°C for 1 hour with  
3     the cells, washed and then goat anti-mouse FITC was added  
5     to the test. The results are presented in Figure 1. The  
7     shaded areas represent the number of cells (Y-axis) with a  
9     given fluorescence intensity (X-axis) as determined by  
11    flow cytometry. KS-14 and TSF-2 exhibit identical  
13    profiles, so KS-14 was used for "addition" experiments.  
15    TSF-4 demonstrated a unique profile, but when added to  
17    KS-14, there was clear additive binding as shown by the  
19    large number of cells accumulating in the highest  
21    fluorescence channel. When P<sub>3</sub>, a control antibody that is  
23    not reactive with SCLC was added, no such "synergy"  
25    occurred.

27     The above in vitro testing procedures demonstrate that  
29    antibody TSF-4 together with either TSF-2 or KS-1/4 may be  
31    used as functionally specific antibodies in accordance  
33    with the present invention.

35

#### 37     EXAMPLE II. Immunoconjugates from Two Antibodies

##### 39     Against Small Cell Lung Cancer

41     The two antibody species TFS-2 and TFS-4 each are  
43    covalently linked to the anti-cancer drug doxorubicin  
45    through a suitable linker molecule. The linker molecule  
47    and procedure described in U.S. Patent Number 4,680,388  
49    may be used. The resulting immunoconjugates are separately  
51    tested for the maximum tolerated dose (MTD) to humans.  
53    This is achieved by administering single or multiple fixed  
55    doses to groups of patients (usually 3-5), monitoring for  
57    toxicity and determining the MTD as the dose level below  
59    which limiting toxicity was reached. Doxorubicin itself  
61    commonly causes reversible bone marrow depression,  
63    alopecia, mucositis and irreversible cardiomyopathy at  
65    cumulative doses in excess of 550 mg/m<sup>2</sup>. When linked to  
67    an antibody for delivery to tumor and possibly normal  
69    cross-reactive tissues, its toxicities would be expected  
71    to be different, and dependent on cross-reactivity. TFS-4,

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1 for example, when linked to doxorubicin may cause neuro-  
toxicity at some dose and TFS-2 doxorubicin may result in  
3 thyroid or pancreatic toxicity. Dosages may be modified  
accordingly.

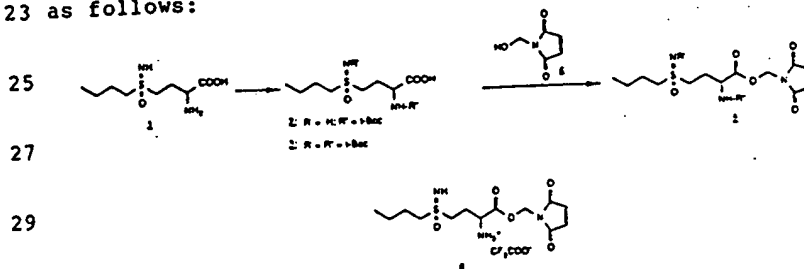
5 Both immunoconjugates are administered near their MTD  
to a patient with SCLC. Both immunoconjugates are expected  
7 to accumulate additively on the tumor cells, without over-  
lapping toxicity toward essential normal tissues.

9

11 **EXAMPLE III. Potentiation of Cytotoxicity Using**  
13 **Immunoconjugates of Two Antibodies Against**  
**Small Cell Lung Cancer**

15 Buthionine sulfoximine (BSO) is a synthetic amino acid  
that inhibits gamma-glutamylcysteine synthetase and leads  
17 to a marked reduction in glutathione (GHS) in cells. BSO  
has been reported to enhance the effectiveness of certain  
19 anti-cancer drugs. BSO is synthesized or is obtained from  
Chemical Dynamics Corporation, South Plainfield, New  
21 Jersey. The BSO is linked to monoclonal antibody TFS-4.

A synthetic scheme for conjugating BSO to antibody is  
23 as follows:



Preparation of N,N'-Bis(t-butoxycarbonyl)buthionine

33 sulfoximine 3: To a stirred solution of buthionine  
sulfoximine (1.6 g, 5 mmol) in THF-H<sub>2</sub>O (1:1, 25 mL) was  
35 added Et<sub>3</sub>N (750 uL, 1.1 equiv) followed by di-tert-  
butylpyrocarbonate (4.5 g, 4.1 equiv). The clear biphasic  
37 mixture was stirred for 15 h. At the end tetrahydrofuran

1 was evaporated in vacuo and MeOH (15 mL), Et<sub>3</sub>N (750  $\mu$ L) were added and to the homogeneous solution were added 3 portions of di-tert-butylpyrocarbonate (15.25 g, 14 equiv, 2 equiv/48 h during the first 96 h and then 5 equiv/48 h 5 during the later 96 h) over a period of 8 days. Reversed phase TLC, MeOH-H<sub>2</sub>O (7:3) showed mainly two spots (RF = 7 0.7 and 0.4) after spraying and heating with ninhydrin. Acetic acid (1 mL) was added to the reaction mixture and 9 volatiles were evaporated in vacuo and the residue reevaporated with toluene in vacuo. The resulting oil was 11 dissolved in MeOH and water was added to slight turbidity. It was then charged onto a C<sub>18</sub> column equilibrated with 13 MeOH-H<sub>2</sub>O (3:7) and eluted with MeOH-H<sub>2</sub>O (3:7, 500 mL), MeOH-H<sub>2</sub>O (2:3, 250 mL), MeOH-H<sub>2</sub>O (1:1, 200 mL), MeOH-H<sub>2</sub>O 15 (3:1, 300 mL) and finally with MeOH (300 mL) collecting fractions of 75 mL size. Fractions containing N-t-butoxy- 17 carbonylbuthionine sulfoximine 2 were combined and evaporated in vacuo to give 1.05 g as a powder. <sup>1</sup>H NMR'H 19 (CDCl<sub>3</sub>)  $\delta$  8.1 (2H, exchangeable with D<sub>2</sub>O, br's), 5.8 (1H, exchangeable with D<sub>2</sub>O, br.s), 4.3 (1H, m), 3.2 (4H, m), 21 2.3 (2H, m), 2.0-0.8 (16 H, m). Fractions containing N,N'-bis(t-butoxycarbonyl)buthionine sulfoximine 3 were 23 combined and evaporated in vacuo to give 450 mg as a foam. <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  6.0-5.5 (2H, exchangeable with 25 D<sub>2</sub>O, br's), 4.40 (1H, m), 3.4 (4H, m), 2.5-2.2 (2H, m), 1.8 (2H, m), 1.48, 1.45 (18 H, 2xS), 0.97 (3H, t, J = 27 7Hz). <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  176.3, 158.9, 156.4, 80.8, 80.3, 51.5 51.3, 48.1, 48.0, 28.3, 28.1, 27.9, 25.5, 24.3, 24.1, 29 21.5, 13.5.

31 Preparation of N,N'-Bis(t-butoxycarbonyl)sulfoximine  
(maleimido)methyl ester 5: A solution of 3 (290 mg, 0.7 33 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (3 mL) under argon was cooled to 0°C and Et<sub>3</sub>N (100  $\mu$ L) was added. After 10 min. isobutyl chloro- 35 formate (120  $\mu$ L) was added dropwise via a syringe. The solution was stored at 0°C under argon for 1 h. A solution 37 of N-hydroxymethylmaleimide 4 (89 mg, 0.7 mmol) in CH<sub>2</sub>Cl<sub>2</sub>

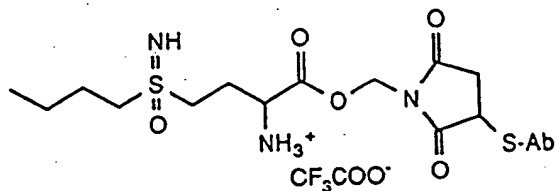
1 (2 mL) then was added dropwise and the amber colored  
suspension was stirred at 0°C for 30 min at which time  
3 TLC, silica gel, MeOH-CH<sub>2</sub>Cl<sub>2</sub> (1:19) indicated completion  
of the reaction. The reaction mixture was diluted with  
5 CH<sub>2</sub>Cl<sub>2</sub> (15 mL) and partitioned between water (10 mL) and  
CH<sub>2</sub>Cl<sub>2</sub>. Organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>), filtered,  
7 evaporated in vacuo. The residue (370 mg) was subjected  
to flash chromatography with MeOH-CH<sub>2</sub>Cl<sub>2</sub> (1:19) on 1 x 15  
9 cm silica gel column. Fractions containing N,N'-bis(t-  
butoxycarbonyl)buthionine sulfoximine (maleimido)methyl  
11 ester 5 were combined and evaporated in vacuo to give a  
pale yellow oil (230 mg, 62%). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 6.8,  
13 (2H, s), 5.6 (2H, m), 5.2 (1H, m), 4.3 (1H, m), 3.5 - 3.1  
(4H, m), 1.5 - 2.1 (2H, m), 1.8 (2H, m), 1.48, 1.45 (18H,  
15 2s), 0.95 (3H, t, J = 7.0 Hz).

Preparation of Sulfoximine (maleimido) methyl ester

17 6: A solution of 5 (71 mg) in CH<sub>2</sub>Cl<sub>2</sub> (350 uL) was treated  
with anhydrous TFA (50 uL). Pale yellow solution was  
19 stored at ambient temperature overnight. TLC, silica gel,  
MeOH-CHCl<sub>3</sub> (1:9) and n-BuOH-AcOH-H<sub>2</sub>O (3:2:1) indicated  
21 completion of the reaction. Volatiles were evaporated.  
The crude product was triturated with Et<sub>2</sub>O (2 x 5 mL) and  
23 washings discarded. <sup>1</sup>H NMR (D<sub>2</sub>O) of the residue showed δ  
6.8 (2H, s), 5.6 (2H, m), 4.6 (1H, m, partly buried under  
25 H<sub>2</sub>O peak), 3.6-3.2 (4H, m), 2.4-2.2 (2H, m), 1.8-1.5 (2H,  
m), 1.4-1.2 (2H, m), 0.8 (3H, t, J = 7.0 Hz) which is  
27 consistent with the proposed structure.

The BSO derivative 6 is conjugated to the monoclonal  
29 antibody TFS-4 (described above). The maleimide group of  
the BSO derivative is reacted with a free sulfhydryl on  
31 the antibody to form the immunoconjugate. The reaction  
procedures are generally as described in U.S. Patent No.  
33 4,659,839. Preferably, the reaction procedure begins with  
isolation of a Fab' fragment from the antibody. This may  
35 be accomplished by conventional procedures; e.g., by first  
treating the antibody with papain to generate a F(ab')<sub>2</sub>  
37 fragment (see Parham et al., J. Immunol. Methods,

1 53:133-173 [1982]). The F(ab')<sub>2</sub> fragment is treated with  
 3 a reducing agent such as dithiothreitol, 2-mercaptoethanol,  
 5 or cysteine under mild reducing conditions to preferen-  
 7 tially cleave the single disulfide bond between the two  
 9 heavy chains without breaking the disulfide bonds between  
 11 heavy and light chains. The two resulting Fab' fragments  
 13 each have one free sulfhydryl group. These Fab' fragments  
 15 are reacted with the derivatized BSO compound in a suitably  
 17 buffered solution under conditions which will not damage  
 19 the antibody fragment. Suitable buffers include such non-  
 21 toxic buffers as sodium phosphate buffer, phosphate  
 23 buffered saline, and sodium bicarbonate buffers, advantage-  
 25 ously at a concentration of about 1.0 M and a pH near  
 27 about 7.0. The resulting immunoconjugate is represented  
 29 by the following formula in which Ab represents the  
 31 antibody fragment:

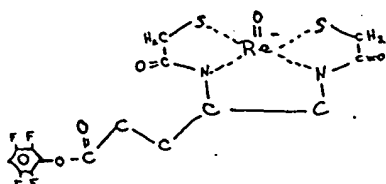


27 Doxorubicin is covalently linked to TFS-2 as described  
 29 in Example II. The TFS-4/BSO is administered to an SCLC  
 31 patient at its maximum tolerated dose, as is the TFS-2/  
 33 doxorubicin. Because BSO can potentiate adriamycin toxi-  
 35 city, and because only the tumor cells and thyroid receive  
 37 both BSO and doxorubicin, the tumor cells should be more  
 39 sensitive to the doxorubicin than any of the normal tissues  
 except thyroid with which the TFS-2 antibody cross-reacts.  
 Possible thyroid failure may be easily monitored by T<sub>4</sub>, T<sub>3</sub>  
 and TSH levels, and replacement hormone completely  
 ameliorates the problems associated with hypothyroidism.

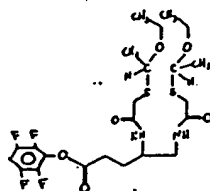


EXAMPLE IV. Immunoconjugates Comprising a  
Radiotherapeutic Compound

A chelate comprising the therapeutically effective  
radionuclide metal  $^{188}\text{Re}$  and having the following  
structural formula is prepared:



Preparation of this chelate is as described in European  
Patent Application Publication Number EP 188,256 or in  
co-pending U.S. Patent Application Serial Number 065,011.  
Sodium perrhenate (3 mL, 15 mCi, produced from a W-188/  
Re-188 research scale generator) was added to a vial  
containing a lyophilized mixture comprising citric acid,  
75 mg; stannous chloride, 0.75 mg; gentisic acid, 0.25 mg;  
and lactose, 100 mg. The vial was agitated gently to mix  
the contents, then incubated at room temperature for 10  
minutes to form a  $^{188}\text{Re}$ -citrate exchange complex. Next,  
0.50 mL of isopropyl alcohol was added to a separate vial  
containing 0.50 mg of 2,3,5,6-tetrafluorophenyl-4,5-bis[S-  
(1-ethoxyethyl)thioacetamido]pentanoate, which is a  
chelating compound comprising ethoxyethyl sulfur protective  
groups and a 2,3,5,6-tetrafluorophenyl ester group, having  
the formula:



1 The vial was agitated for two minutes to completely  
2 dissolve the chelating compound. Next, 0.30 mL of this  
3 solution was transferred to the vial containing the  
4  $^{188}\text{Re}$ -citrate complex prepared above. After gentle  
5 mixing, the vial was incubated in a  $75^{\circ}\text{C} \pm 2^{\circ}\text{C}$  water bath  
6 for 15 minutes, then immediately transferred to a  $0^{\circ}\text{C}$  ice  
7 bath for two minutes. The yields of  $^{188}\text{Re}$ -labeled chelate  
8 then ranged between 75% and 90% as measured by reversed  
9 phase  $\text{C}_{18}$  HPLC analysis.

10 A column containing a  $\text{C}_{18}$  reversed phase low-pressure  
11 material (Baker  $\text{C}_{18}$  cartridges) was used to purify the  
12  $^{188}\text{Re}$ -labeled chelate. After conditioning of the cartridge  
13 with ethanol and water, the sample was loaded and washed  
14 with three times 2 mL of water and three times 2 mL of 20%  
15 ethanol/0.01 M phosphate buffer. The column was then dried  
16 in vacuo and eluted with two times 1.0 mL acetonitrile.  
17 About 75% of the  $^{188}\text{Re}$ -radioactivity was recovered in  
18 greater than 95% purity as the ester chelate compound.  
19 The organic solvent was then evaporated under a flow of  
20 inert gas.

21 The chelate is then conjugated to a Fab fragment of  
22 monoclonal antibody TFS-2 and a Fab fragment of TFS-4 in  
23 separate reaction mixtures. The Fab fragments are  
24 generated by papain treatment according to conventional  
25 procedures.

26 A buffered solution of the antibody fragment (5 mg/mL,  
27 0.5 mL) is added to the purified  $^{188}\text{Re}$ -labeled chelate,  
28 followed by 0.5 mL of 0.5 M carbonate/bicarbonate buffer  
29 pH 9.50. The reaction is kept at room temperature for 15  
30 minutes, then 25 mg of L-lysine, 0.1 mL, is added and the  
31 reaction is pursued at room temperature for 15 minutes  
32 more.

33 A column containing Sephadex G-25 material is used to  
34 purify each  $^{188}\text{Re}$ -labeled immunoconjugate. The reaction  
35 mixture is loaded on top of the column, and 1.2 mL aliquots  
are collected using PBS buffer to rinse the reaction vial

1 and elute the  $^{188}\text{Re}$  immunoconjugate in the third and  
fourth fractions.

3 The immunoconjugate is then further diluted with PBS,  
and radioactivity is measured prior to injection of both  
5 immunoconjugates into an SCLC patient. The two immuno-  
conjugates should accumulate additively only on SCLC and  
7 thyroid tissues within the patient, and the additive thera-  
peutic dosage of both immunoconjugates is selectively  
9 directed to the target tissue.

Current data suggests that doses of B-emitting radio-  
11 nuclides linked to antibodies will be limited by their  
effects on the bone marrow. One approach to overcome this  
13 problem is to harvest and store the marrow prior to treat-  
ment. The second target organ of toxicity will then be  
15 related to the cross-reactivity of the antibody and the  
molecular species (whole antibody,  $\text{F(ab')}_2$ , Fab or Fv)  
17 used. Doses up to 400 mCi  $^{131}\text{I}$  on whole ant body have  
been safely administered when marrow is harvested and  
19 stored for re-infusion.

Radiosensitizers such as misonidazole and BSO (see  
21 Example III) may be used to potentiate the cytotoxicity of  
radionuclides on tumors just as they can drugs. If  
23 suitable potentiation occurs, the radiation dose admin-  
istered may be decreased and bone marrow spared. Radio-  
25 nuclides would be coupled to one antibody and the  
sensitizer would be coupled to the other antibody in the  
27 pair.

29  
31 **EXAMPLE V. Immunoconjugates Comprising a  
Radiodiagnostic Compound**

33 The chelating compound shown in Example IV may be  
radiolabeled with the metal radionuclide  $^{99\text{m}}\text{Tc}$ , a  
35 diagnostic agent, as described in EP 188,256 or USSN  
065,011.

1 One mL of sterile water for injection was added to a  
sterile vial containing a stannous gluconate complex (50  
3 mg sodium gluconate and 1.2 mg stannous chloride dihydrate,  
available from Merck Frosst, Canada, in dry solid form).  
5 and the vial was gently agitated until the contents were  
dissolved. A sterile insulin syringe was used to inject  
7 0.1 mL of the resulting stannous gluconate solution into  
an empty sterile vial. Sodium pertechnetate (0.75 mL,  
9 75-100 mCi, eluted from a  $^{99}\text{Mo}/^{99}\text{Tc}$  generator purchased  
from duPont, Medipysics, Mallinckrodt or E.R. Squibb) was  
11 added, and the vial was agitated gently to mix the contents  
then incubated at room temperature for 10 minutes to form  
13 a  $^{99\text{m}}\text{Tc}$ -gluconate complex.

A separate vial containing 0.3 mg of the chelating  
15 agent in dry solid form was prepared by dispensing a  
solution of 0.3 mg chelating agent in acetonitrile into  
17 the vial, then removing the solvent under  $\text{N}_2$  gas. To this  
vial was then added 0.87 mL of 100% isopropyl alcohol, and  
19 the vial was gently shaken for about two minutes to com-  
pletely dissolve the chelating agent, which was 2,3,5,6-  
21 tetrafluorophenyl 4,5-bis[S-(1-ethoxyethyl)thioacetamido]-  
pentanoate. Next, 0.58 mL of this solution of the  
23 chelating agent was transferred to a vial containing 0.16  
mL of glacial acetic acid / 0.2 N HCl (2:14), and the vial  
25 was gently agitated. Of this acidified solution, 0.5 mL  
was transferred to the vial containing the  $^{99\text{m}}\text{Tc}$ -gluconate  
27 complex, prepared above. After gentle agitation to mix,  
the vial was incubated in a  $75^\circ\text{C} \pm 2^\circ\text{C}$  water bath for 15  
29 minutes, then immediately transferred to a  $0^\circ\text{C}$  ice bath  
for two minutes.

31 To a separate vial containing 10 mg of the Fab  
fragment of a monoclonal antibody (TSF-2 or TSF-4 as  
33 described in Example IV) in 0.5 mL of phosphate buffered  
saline, is added 0.37 mL of 1.0 M sodium bicarbonate  
35 buffer, pH 10.0. The vial is gently agitated.

The vial containing the acidified solution of the  
37  $^{99\text{m}}\text{Tc}$ -labeled chelate (see above) is removed from the ice

1 bath, 0.1 mL of the sodium bicarbonate buffer is added,  
and the vial is agitated to mix. Immediately, the  
3 buffered antibody solution (above) is added, gently  
agitated to mix and incubated at room temperature for 20  
5 minutes to allow conjugation of the radiolabeled chelate  
to the antibody.

7 A column containing an anion exchanger, either DEAE-  
Sephadex or QAE-Sephadex, is used to purify each of the  
9 two immunoconjugates. The column is prepared under  
aseptic conditions as follows. Five 1 mL QAE-Sephadex  
11 columns are connected to form a single column. Alterna-  
tively, a single 5 mL QAE-Sephadex column may be used.  
13 The column is washed with 5 mL of 37 mM sodium phosphate  
buffer, pH 6.8. A 1.2 u filter (available from Millipore)  
15 is attached to the column, and a 0.2 u filter is attached  
to the 1.2 u filter. A 22-gauge sterile, nonpyrogenic  
17 needle is attached to the 0.2 u filter.

The reaction mixture is drawn up into a 3 mL or 5 mL  
19 syringe, and any air bubbles are removed from the solution.  
After removal of the needle, the syringe is connected to  
21 the QAE-Sephadex column on the end opposite the filters.  
The needle cap is removed from the 22-gauge needle attached  
23 to the filter end of the column and the needle tip is  
inserted into a sterile, nonpyrogenic test tube. Slowly,  
25 over two minutes, the reaction mixture is injected into  
the column. The eluant collected in the test tube is  
27 discarded. The now empty syringe on top of the column is  
replaced with a 5 mL syringe containing 5 mL of 75 mM  
29 (0.45%) sodium chloride solution (from which air bubbles  
have been removed.) The needle at the other end of the  
31 column is inserted aseptically into a sterile,  
nonpyrogenic 10 mL serum vial. Slowly, over two minutes,  
33 the NaCl solution is injected into the column, and the  
eluent is collected in the serum vial.

35 A total radioactivity in the serum vial is measured  
using a dose calibrator. The contents of both serum vials  
37 are combined and drawn up into a sterile, pyrogen-free,

1 30 cc syringe and diluted to a total volume of 30 mL with  
sterile 0.9% NaCl for injection of each immunoconjugates  
3 into a human SCLC patient sequentially over a few days.  
The two radiodiagnostic agent-bearing antibodies should  
5 accumulate additively on the target cancer tissue and on  
thyroid tissue.

7 One major benefit of using the two radiodiagnostic  
agents, compared to a single antibody, is to detect meta-  
9 stases expressing only one of the antigens in sufficient  
abundance to accumulate the antibody. With negligible  
11 overlapping cross-reactivity on normal tissues, the two  
antibodies may also distinguish real foci of tumor (both  
13 tests are positive) from normal tissue accumulation (only  
1 test positive). The known uptake of the tracer into  
15 highly vascularized areas, or into kidneys if Fab  
fragments are employed, needs to be considered in this  
17 evaluation.

## THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A composition comprising two or more different antibody species, each having a diagnostic or therapeutic agent attached thereto, wherein each of said antibody species is reactive with a different epitope on a target site and wherein the patterns of cross-reactivity for each antibody species are non-overlapping, for use within a method for delivering one or more diagnostic or therapeutic agents to a target site within a mammalian or human host.

2. The composition of claim 1 wherein an identical diagnostic or therapeutic agent is attached to each of the antibody species.

3. The composition of claim 2 wherein the diagnostic agent is a diagnostically effective radionuclide.

4. The composition of claim 3 wherein the diagnostic agent is selected from the group consisting of  $^{99m}\text{Tc}$ ,  $^{131}\text{I}$ ,  $^{111}\text{In}$ , and  $^{123}\text{I}$ ,  $^{76}\text{Br}$ , and  $^{18}\text{F}$ .

5. The composition of claim 3 wherein the diagnostic agent is  $^{99m}\text{Tc}$  in the form of a chelate.

6. The composition of claim 1 wherein the therapeutic agent is  $^{188}\text{Re}$  in the form of a chelate.

7. The composition of claim 1 wherein different therapeutic agents are attached to each of the antibody species.

8. The composition of any of claims 1, 2 or 7 wherein each therapeutic agent is selected from the group consisting of therapeutically effective radionuclides, drugs, toxins, sensitizers, and biological response modifiers.

9. The composition of claim 8 wherein the radionuclide is selected from the group consisting of  $^{188}\text{Re}$ ,  $^{186}\text{Re}$ ,  $^{203}\text{Pb}$ ,  $^{212}\text{Pb}$ ,  $^{212}\text{Bi}$ ,  $^{109}\text{Pd}$ ,  $^{64}\text{Cu}$ ,  $^{67}\text{Cu}$ ,  $^{131}\text{I}$ ,  $^{211}\text{At}$ ,  $^{97}\text{Ru}$ ,  $^{105}\text{Rh}$ ,  $^{198}\text{Au}$ , and  $^{199}\text{Ag}$ .

10. The composition of claim 8 wherein the toxin is selected from the group consisting of ricin, abrin, diphtheria toxin, Pseudomonas exotoxin A, ribosomal inactivating proteins, mycotoxins, trichothecenes, and therapeutically effective fragments thereof.

11. The composition of claim 8 wherein the target site is a cancer site and the drug is an anti-cancer drug.

12. The composition of claim 11 wherein the drug is an anti-cancer antibiotic.

13. The composition of claim 7 wherein the target site is a cancer site, a sensitizing drug is attached to one antibody species, and a therapeutically effective radionuclide is attached to a second antibody species.

14. The composition of claim 7 wherein the target site is a cancer site, a sensitizing drug is attached to one antibody species, and an anti-cancer drug is attached to a second antibody species.

15. The composition of claim 1 wherein the target site is a cancer site.

16. The composition of claim 1 wherein each of the antibody species is a monoclonal antibody or a fragment thereof.



17. The composition of claim 16 wherein each antibody species is a monoclonal antibody, or a fragment thereof, which binds to cancer cells.

18. The composition of any of claims 1, 2, 5, 6, 7 or 17 wherein one antibody species is monoclonal antibody NR-LU-10 or a fragment thereof, and a second antibody species is NR-LU-11 or a fragment thereof.

19. A kit for diagnostic or therapeutic use, comprising two or more antibody species, wherein each of said antibody species is reactive with a different epitope on a target site and the patterns of cross-reactivity for each of said antibody species are non-overlapping.

20. A kit for diagnostic or therapeutic use comprising two separate compositions, each composition comprising at least one antibody species having a diagnostic or therapeutic agent attached thereto, wherein each of said antibody species is reactive with a different epitope on a target site, and wherein the patterns of cross-reactivity for each antibody species are substantially non-overlapping, for administration to a single mammalian or human host.

21. The use of two or more antibody species having a diagnostic or therapeutic agent attached thereto, wherein each of said antibody species is reactive with a different epitope on a target site, and wherein the patterns of cross-reactivity for each antibody species are substantially non-overlapping, in the manufacture of a composition for delivering one or more diagnostic or therapeutic agents to a target site within a mammalian or human host.

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22. A composition of claim 1, a kit of claim 19 or 20, or a method of use of claim 21, substantially as hereinbefore described with reference to the accompanying examples.

23. The steps, features, compositions and compounds disclosed herein or referred to or indicated in the specification and/or claims of this application, individually or collectively, and any and all combinations of any two or more of said steps or features.

DATED this SEVENTEENTH day of MARCH 1989

NeoRx Corporation

by DAVIES & COLLISON  
Patent Attorneys for the applicant(s)

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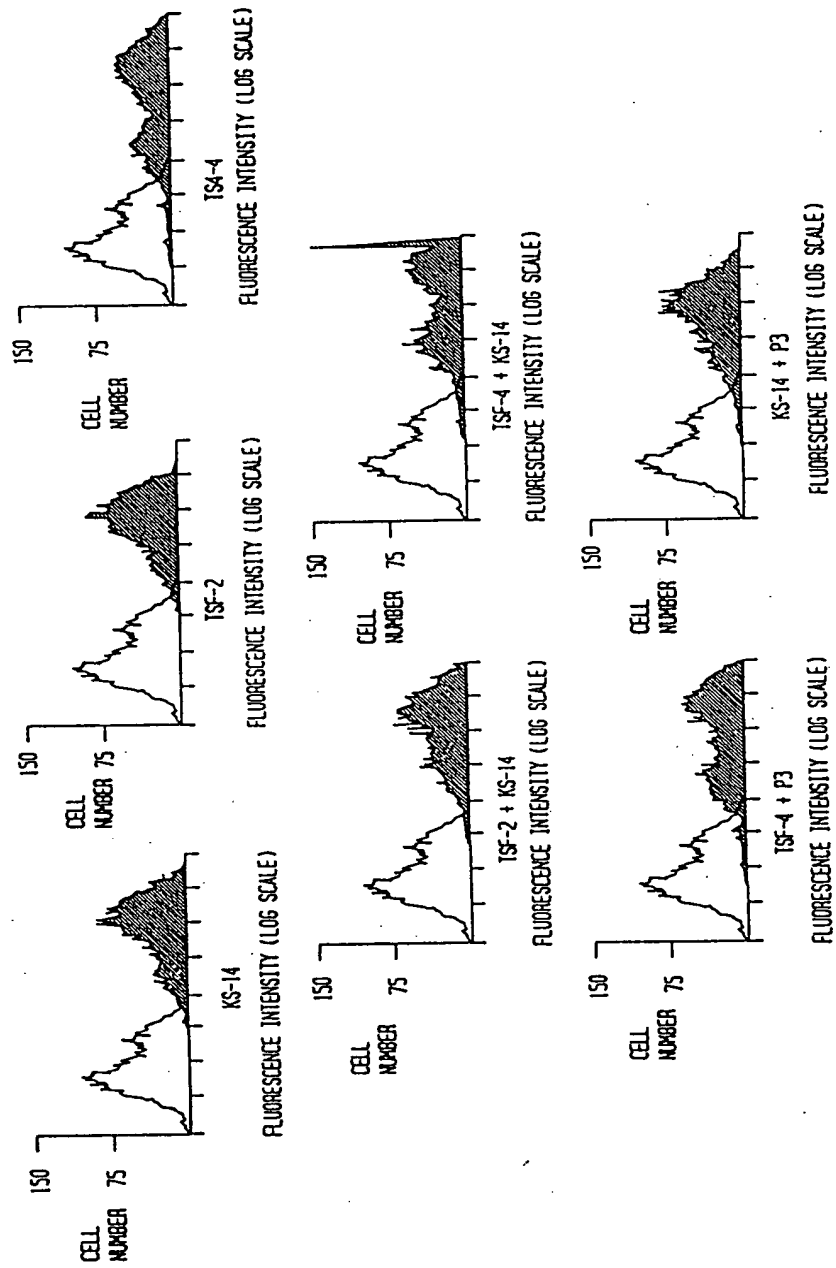


FIGURE 1

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